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The hepatitis B viral X protein activates NF-κB signaling pathway through the up-regulation of TBK1

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ABSTRACT

The hepatitis B viral X protein (HBx) is a multifunctional protein that plays a role in the activation of the NF-κB signaling pathway. In this study, we demonstrate that HBx up-regulates expression of TANK-binding kinase-1 (TBK1) at the transcriptional level. Increased levels of TBK1 protein then induce the phosphorylation of NF-κB p65 at serine 536. Because TBK1 is up-regulated by HBx as part of its promotion of NF-κB activation, it is possible that increased TBK1 levels play a role in the development of hepatocellular carcinoma (HCC). From these data, we suggest that TBK1 may play a role in the HBx-mediated activation of the NF-κB signaling pathway and the development of HCCs.

1. Introduction

Hepatitis B virus (HBV) is the major worldwide etiologic factor of chronic liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC), especially in Asia and Africa [1]. One of the pathogenic mechanisms associated with HBV infection is the activation of the NF-κB signaling pathway, a key regulator of cancer development.

The HBV genome encodes four genes, HBc, HBs, HBV pol and HBx [2]. Our group and several others have reported that HBV and HBx [3–6] can activate NF-κB in liver cells. This finding correlates with studies that have reported that HBx plays a variety of roles in host cells, which includes a role as a promiscuous transactivator that is involved in cell growth [7] and cell death [8] in HCC.

Recently, TANK-binding kinase-1 (TBK1) was found to be structurally similar to the classical IKKα, β kinases [9]. TBK1 is a key component in the development of the IFN-mediated antiviral response. For example, TBK1 phosphorylates transcription factors such as IFN regulatory factor (IRF)-3 [10] and IRF-7 [11], leading to the activation of the IFNαβ gene. Furthermore, recent studies have shown that TBK1 is involved in NF-κB activation. TBK1 is among the kinases that mediate the inducible phosphorylation of p65 at Ser-536, an event that is proposed to stimulate inherent p65 transactivation and thus NF-κB signaling [12].

In this study, we demonstrate that TBK1 is expressed strongly in liver cancer cells and that it is involved in regulating HBx-mediated NF-κB activity by controlling basal/constitutive p65/RelA phosphorylation at Ser-536, a modification that is proposed to contribute to the transactivation of NF-κB. These data indicate a role for TBK1 in controlling proliferation of certain liver cancer cells through the regulation of constitutive HBx-mediated NF-κB activity.

2. Materials and methods (see Supplementary data)

2.1. Plasmids

The wild-type TBK1 expression plasmid was a kind gift from Kate Fitzgerald (University of Massachusetts Medical School). The TBK1 K38A mutant was cloned by PCR-based mutagenesis. TBK1 KD was cloned into the eukaryotic expression vector p3XFlag-CMV7.1 (Sigma, St. Louis, MO). For other plasmids, see Supplementary data.

2.2. Cell culture

To establish Huh7 lines stably expressing HBx, we inserted the HBx complementary DNA sequence (HBV *adr* type) into the pRcCMV (Invitrogen, Carlsbad, CA) vector and transfected the

Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B viral X protein; NF-κB, nuclear factor-kappa B; TBK1, TANK-binding kinase-1; HCC, hepatocellular carcinoma

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Huh7 cells with pRcCMV (Huh7-cont) as a control and pRcCMV/HA-HBx (Huh7-HBx) by using Fugene6 (Roche, Penzberg, Germany). G418 (Invitrogen, Carlsbad, CA) was used to select colonies. For other cell lines, see Supplementary data.

3. Results

3.1. Establishment of a cell line that stably expresses HBx

In order to identify new factors involved in the HBx-mediated NF-κB signaling pathway, we established human hepatoma cell lines that stably express HBx protein (Huh7_HBx) and control cells (Huh7_cont), which only contain the empty expression vector. In our cell lines, stable expression of HBx transcript was confirmed by RT-PCR analysis (Fig. 1a). We also confirmed that Huh7_HBx cell lines were capable of activating the NF-κB pathway by detecting a significant increase in IκBα degradation (Fig. 1b) and observing increased NF-κB2 (p52) expression (Fig. 1c). Using an NF-κB phospho-specific antibody microarray, we showed higher levels of phosphorylation of p65 Ser-468 and Ser-536 in Huh7_HBx cell lines compared to Huh7_cont cell lines (Fig. 1d). We were also able to observe similar phosphorylation of p65 Ser-536 by western blot analysis (Fig. 1e). Therefore, these results suggest that the NF-κB pathway was activated in our HBx-expressing cell line.

3.2. HBx-induced elevation of TBK1 expression causes NF-κB activation

As a preliminary step towards investigating how HBx activates the NF-κB pathway, we used a Human NF-κB Signaling Pathway RT^{2}Profiler™ PCR Array (SABiosciences Corporation) to compare transcript levels in the control and HBx-expressing cell lines. Using this array, we were able to observe the transcript levels of 84 genes involved in the NF-κB pathway (see Supplementary Table 1).

Based on differences in expression, we chose 12 of the most highly up-regulated genes for further examination by real-time RT-PCR (Fig. 2a). One of the more interesting candidates was TBK1 because this protein plays a direct role in activating NF-κB by phosphorylating IκB, which allows NF-κB to translocate to the nucleus. Real-time RT-PCR confirmed that TBK1 transcript levels were 5.7 times higher in the HBx cell line than in the control. Furthermore, we were able to observe a similar up-regulation of TBK1 protein levels using western blot analysis (Fig. 2b). In immunoprecipitation experiments, we did not observe any evidence for an interaction between HBx and TBK1 (data not shown), further demonstrating that the increase in TBK1 protein was due to increased transcription that was mediated by HBx. To determine if HBx regulated the expression levels of other IKK-related kinases in our system, we examined the expression levels of IKKα and IKKε by western blot analysis (Fig. 2b).

Interestingly, we found that IKKα was up-regulated while IKKε was down-regulated. It is currently unknown how IKKε affects HBx-mediated NF-κB activation. However, IKKα has been previously reported to play a role in HBV-mediated NF-κB activation [13] and we were able to confirm an increase in IKKα kinase activity in Huh7_HBx cells (Fig. 2c).

Taken together, these results indicate that our HBx cell line is a valid model for studying HBx-mediated NF-κB activation and that TBK1 may mediate HBx up-regulation of the NF-κB pathway.

To determine whether TBK1 might play a role in the phosphorylation of p65 Ser-536, we performed a TBK1 knockdown experiment using TBK1 shRNA. Following transfection of Huh7_ cont and Huh7_HBx cells with TBK1 shRNA, we observed a decreased level of expression of phosphorylated p65 Ser-536 (Fig. 2d). However, no changes in expression of IKKε and IKKα were detected, which suggests that the down-regulation of phosphorylated p65 was related specifically to the down-regulation of TBK1. Thus, we have been able to show that both TBK1 mRNA and protein levels are up-regulated in our Huh7_HBx cell line and that TBK1 levels are closely linked to phosphorylation of NF-κB p65 at Ser-536.

3.3. TBK1 is involved in the HBx-mediated NF-κB pathway

Next, we examined whether TBK1 plays a role in HBx-mediated activation of the NF-κB pathway. We transiently transfected sev-

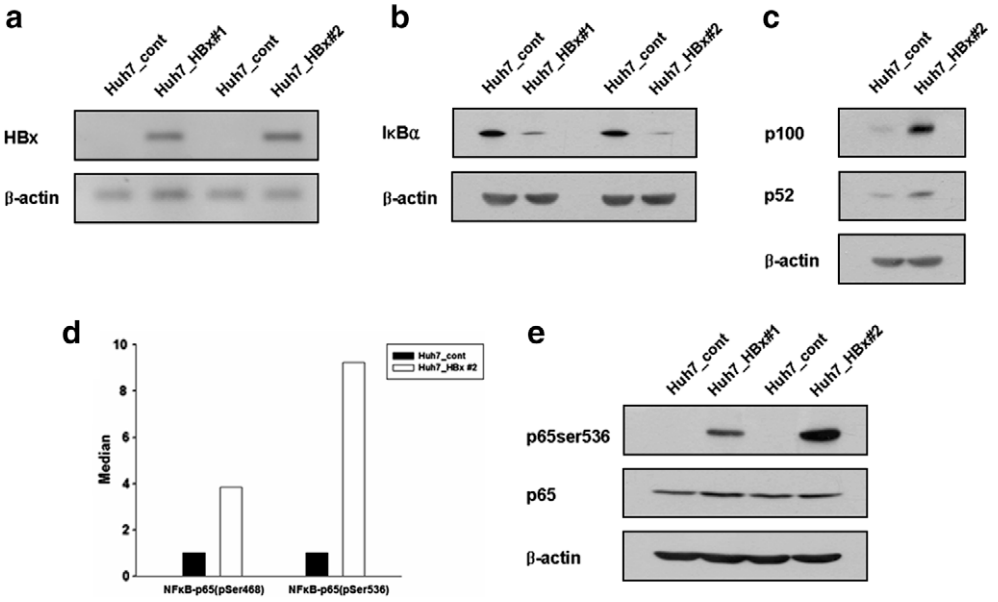


Fig. 1. Examination of NF-κB activity in a cell line stably expressing HBx. (a) Expression of the HBx transcript was assessed by RT-PCR. Total RNA was extracted and cDNA was synthesized by priming with oligo dTs. DNA fragments encoding HBx and β-actin were amplified with gene-specific primers and PCR products were resolved on an agarose gel. (b) Endogenous levels of IκBα protein in Huh7_cont and Huh7_HBx cells were determined by immunoblot. β-Actin was used as a loading control. (c) Immunoblot analyses of NF-κB2 (p52), (p100) and β-actin levels in Huh7_cont and Huh7_HBx cells. Immunoblots were performed using specific antibodies against the proteins indicated. (d) Phosphorylation of p65 at Ser-468 and Ser-536 was analyzed using a NF-κB phospho-specific antibody microarray. (e) Levels of p65 phosphorylated at Ser-536, p65 and β-actin were analyzed by immunoblot using antibodies against p65 phosphorylated at Ser-536, p65 and β-actin.

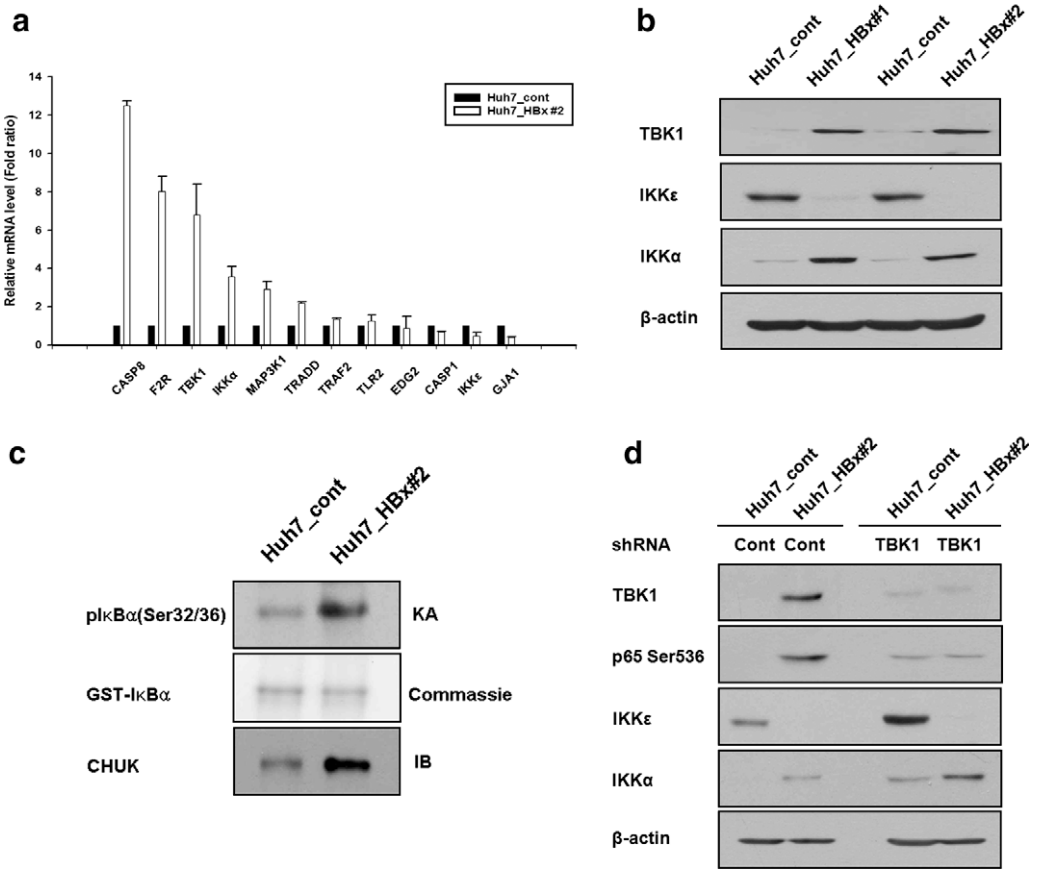


Fig. 2. Identification of TBK1 in Huh7_cont and Huh7_HBx cell lines. (a) Real-time RT-PCR analysis was performed to determine the levels CASP8, F2R, TBK1, IKKα, MAP3K1, TRADD, TRAF2, TLR2, EDG2, CASP1, IKKε and GJA1, in Huh7_cont and Huh7_HBx cells. All numerical data represent the mean and standard deviation of three independent experiments. (b) Expression of TBK1, IKKε, IKKα and β-actin, in Huh7_cont and Huh7_HBx cells. Immunoblot analysis was performed with anti-TBK1, IKKε, IKKα and β-actin antibodies. (c) Huh7_cont and Huh7_HBx cell lysates were immunoprecipitated with antibody against IKKα. One set of immunoprecipitates was subjected to the kinase assay (KA), which uses GST-IκBα (Coomassie stain) as a substrate. The other set of immunoprecipitates was subjected to 7.5% SDS–PAGE and analyzed by immunoblotting (IB) with the anti-IKKα Ab. The abundance of immunoprecipitated kinase complex was confirmed by immunoblotting against IKKα. (d) Huh7_cont and Huh7_HBx cells were transfected with control and TBK1 shRNA and subjected to puromycin selection. The expression of TBK1, p65 Ser-536, IKKε, IKKα and β-actin, in Huh7_cont and Huh7_HBx cells, was examined by immunoblot analysis with anti-TBK1, p65 Ser-536, IKKε, IKKα and β-actin antibodies.

eral human hepatoma (Huh7 and Hep3B) and human embryo kidney (HEK293 and HEK293T) cell lines with a mammalian overexpression plasmid containing TBK1 cDNA. Using a luciferase reporter assay to detect transcription of NF-κB, we found that overexpression of TBK1 increased NF-κB activity to different levels in all cell lines (Fig. 3a).

To establish a relationship between HBx and TBK1 in the activation of NF-κB, we constructed two TBK1 mutants; TBK1 KD, in which the TBK1 kinase domain was deleted and TBK1 K38A, the point mutant. Both mutants have been previously reported to be dominant negative forms that inhibit the activity of wild-type TBK1 [9]. Using the luciferase reporter assay, we observed that co-transfection of cells with vectors overexpressing TBK1 and HBx synergized a further increase in NF-κB activity, compared to TBK1 or HBx expression alone. However, both TBK1 KD and TBK1 K38A inhibited HBx-mediated NF-κB activation in HEK293 cells (Fig. 3b).

Many previous studies have shown that HBx activates NF-κB by two- to fivefold [13,14]. In this study, HBx increases NF-κB activity by 2.4-fold in HEK293 cells (Fig. 3b).

Furthermore, the TBK1 K38A mutant inhibited HBx-mediated phosphorylation of p65 at Ser-536 in HEK293T cells (Fig. 3c).

We also performed immunofluorescence analyses to determine if the expression of HBx or TBK1 could induce nuclear translocation of p65, an important step that must occur before p65 can bind DNA. We transiently transfected HEK293T cells with Myc-p65, HA-HBx, Flag-TBK1 and Flag-TBK1 K38A. Overexpression of HA-HBx and Flag-TBK1 resulted in the translocation of the p65 subunit of NF-κB (Myc-p65) into the nucleus, whereas it localized to the cytoplasm with overexpression of the dominant negative mutant Flag-TBK1 K38A (Fig. 3d).

Previous reports have shown that TBK1-mediated NF-κB activation can be blocked by dominant negative (DN) versions of NIK, IKKα and IKKβ [9]. Therefore, we used the luciferase assay to determine whether IKKα DN or NIK DN could inhibit reporter stimulation when TBK1 is overexpressed. We found that both DN mutants were capable of inhibiting TBK1-mediated NF-κB activation in HEK293 cells (Fig. 3e).

Activation of the NF-κB gene program requires DNA-binding by Rel family proteins. Previously, TBK1 was reported to induce p50:p65 DNA-binding activity in HEK293 cells [9]. Supershift EMSA was used to explore the HBx-mediated increase in NF-κB DNA-binding activity in the Huh7_HBx cell line. Only NF-κB p65 could be identified in the NF-κB-DNA complex induced by HBx and TBK1 (Fig. 3f).

Our data suggest that in the Huh7_HBx cell line, the up-regulation of TBK1 is able to promote NF-κB activation.

3.4. TBK1 expression level increases in cancer cell lines and tumor tissue samples

A positive correlation between NF-κB activation and the progression of cancer has been widely reported. Based on our observa-

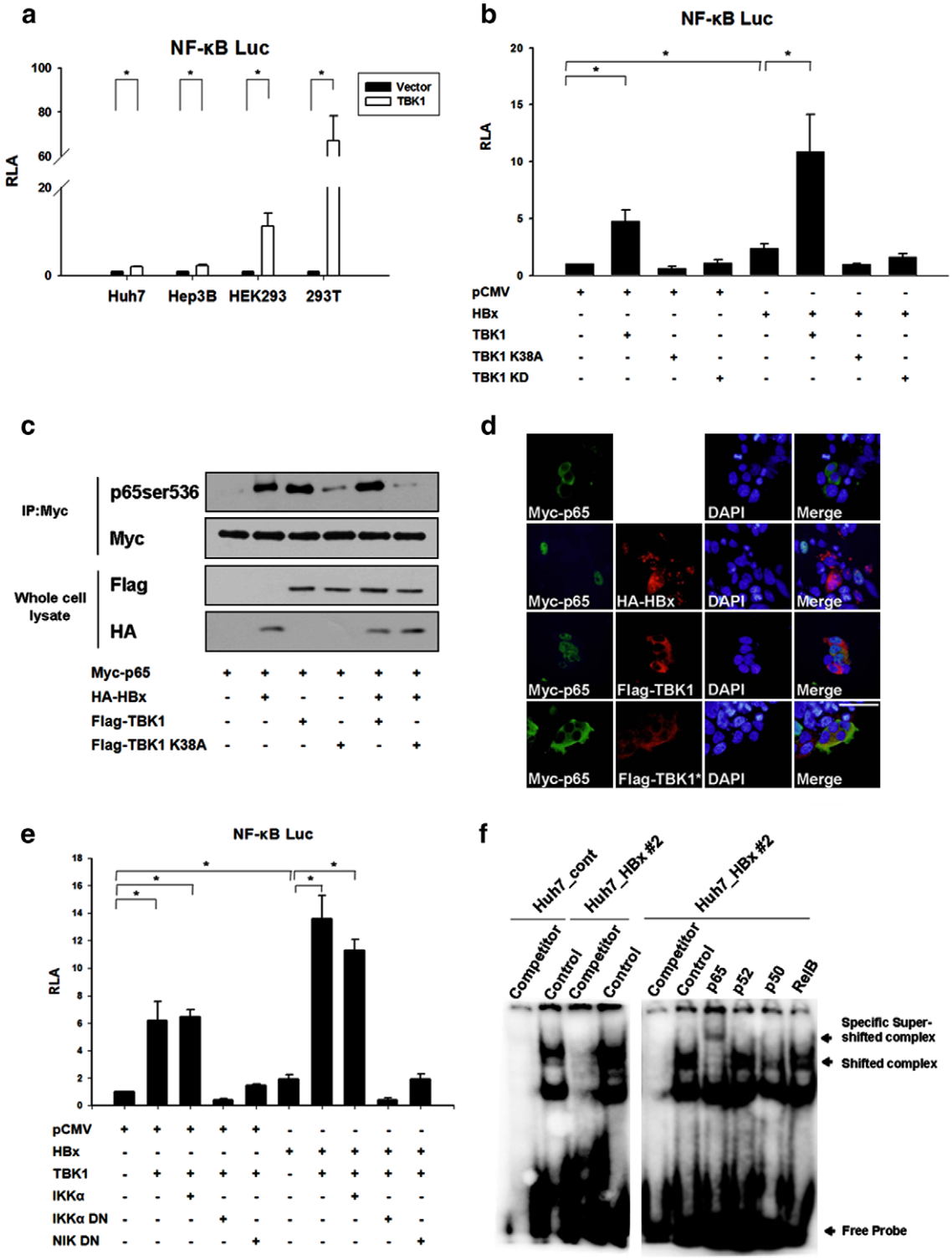


Fig. 3. Analysis of NF-κB activation by HBx and TBK1. NF-κB activation was analyzed by a luciferase reporter assay. All cells were co-transfected with 0.25 μg pNF-κB-Luc reporter plasmid and 0.05 μg pRL-SV40. Additional expression vectors were transfected as indicated. (a) Following transfection of Huh7, Hep3B, HEK293 and HEK293T cells with 0.5 μg pCDNA3-Flag/TBK1 (TBK1 expression plasmid), luciferase activities were estimated. (b) HEK293 cells were co-transfected with 0.5 μg pRcCMV/HA-HBx and 0.5 μg p3XFlag-CMV/TBK1, p3XFlag-CMV/TBK1 K38A or p3xFlag-CMV/TBK1 KD. The luciferase assay was performed 48 h after transfection. (c) HEK293T cells were co-transfected with pCMV-myc/p65 and different combinations of p3XFlag-CMV/TBK1 or p3XFlag-CMV/TBK1 K38A and pRcCMV/HA-HBx. With the exception of pCMV-myc/p65, all of the other plasmids have the SV40 ori. After 48 h, cell lysates were prepared and immunoprecipitated using the anti-myc antibody. Phosphorylation of NF-κB p65 at the Ser-536 residue and expression of TBK1, TBK1 K38A, HBx and p65, were tested by immunoblot analysis with anti-phospho p65 Ser-536, Flag, HA and Myc antibodies. (d) HEK293T cells were co-transfected with pCMV-myc/p65 (Myc-p65) and p3XFlag-CMV/TBK1 (Flag-TBK1) or p3XFlag-CMV/TBK1 K38A (Flag-TBK1*) and pRcCMV/HA-HBx (HA-HBx). After 24 h, cellular localization of p65 (green), HBx (red), TBK1 (red) and TBK1 mutant (red) were examined. Scale bar, 50 μm. (e) HEK293 cells were co-transfected with various combinations of 0.5 μg pRcCMV/HA-HBx, p3XFlag-CMV/TBK1, pFlag-IKKα, pFlag-IKKα DN and pFlag-NIK DN. After 48 h, luciferase assays were performed. (f) EMSA analysis of the effect of HBx-mediated NF-κB activation in Huh7_cont and Huh7_HBx cells. Supershift analysis of NF-κB/*NF-κB probe complexes using anti-p50, p52, p65 and RelB antibodies (Ab), as described in Materials and Methods. Data represent the mean ± standard error of the mean. $^{*}P < 0.05$.

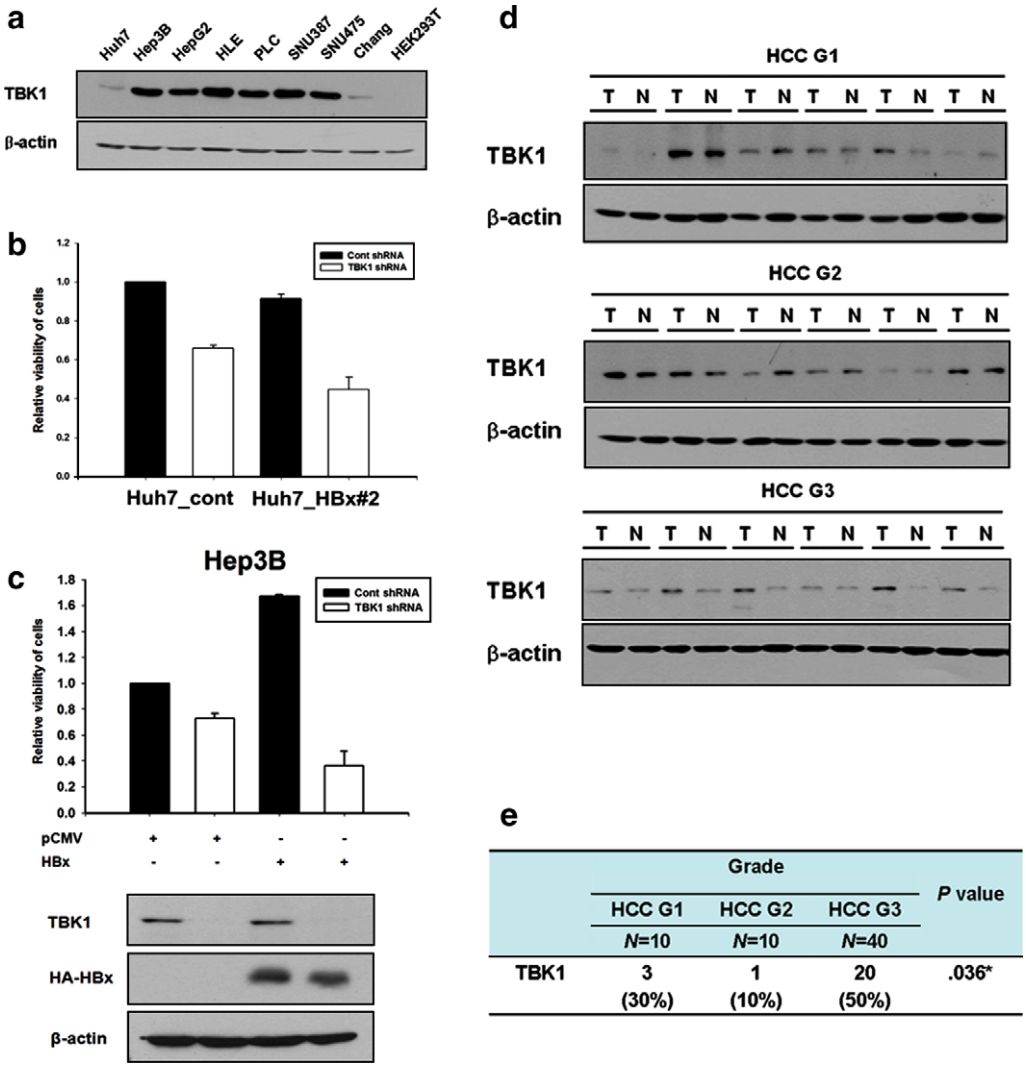
tions that NF-κB activity increased with the level of TBK1, we postulated that cancer cells and tumor tissues would exhibit elevated TBK1 protein levels. We performed western blot analyses to measure the concentration of TBK1 in human hepatoma (Huh7, Hep3B, HepG2, HLE, PLC, SNU387 and SNU475), Chang liver and human embryo kidney (HEK293T) cell lines. We discovered significantly higher TBK1 levels in human hepatoma cell lines (Hep3B, HepG2, HLE, PLC, SNU387 and SNU475) than in Chang liver and HEK293T cell lines (Fig. 4a).

Because NF-κB activity is known to promote proliferation [15], knockdown of TBK1 may decrease cell viability. We found that TBK1 shRNA knockdown of TBK1 levels down-regulated cell proliferation more in Huh7_HBx cells than in Huh7_cont cells (Fig. 4b). Also, we confirmed that TBK1 shRNA knockdown of TBK1 levels down-regulated cell proliferation in Hep3B cells (Fig. 4c).

As chronic infection with HBV is closely linked to HCC, we tested to see if HBV-positive serum from HCC tissue samples had increased levels of TBK1. Immunoblot analysis of TBK1 levels was performed on 75 human HCC tissue samples. A significant increase in TBK1 expression was observed in tumor versus non-tumor tissues. The relationship between expression of TBK1 and HCC grade was analyzed by calculating the Spearman correlation and a *P* value of 0.036 was considered statistically significant. Analysis revealed a strong correlation between increased TBK1 expression and grade 3 HCC (Fig. 4d and e and see Supplementary Table 2).

4. Discussion

In this study, we proposed that HBx activate NF-κB signaling through the up-regulation of TBK1. We demonstrated that HBx may up-regulate the expression of TBK1 at the transcriptional level by examining HBx-expressing cell lines using a Human NF-κB Signaling Pathway PCR Array, real-time RT-PCR analysis and western blot analyses (Fig. 2a and b and see Supplementary Table 1). We also observed that TBK1 may be responsible for HBx-mediated NF-κB activation by using dominant negative forms of TBK1. Specifically, these mutants inhibited HBx-mediated NF-κB-dependent promoter activity (Fig. 3b), as well as phosphorylation of NF-κB p65 at Ser-536 (Fig. 3c). Furthermore, we found elevated TBK1 expression levels in cancer cell lines and HBV-positive human



e

	Grade			*P* value
	HCC G1	HCC G2	HCC G3	
	N=10	*N*=10	*N*=40	
TBK1	3 (30%)	1 (10%)	20 (50%)	.036*

Fig. 4. TBK1 is up-regulated in HCC. (a) Expression of TBK1 and β-actin in Huh7, Hep3B, HepG2, HLE, PLC, SNU387, SNU475, Chang and HEK293T cells. Immunoblot analysis was performed using anti-TBK1 or β-actin antibodies. (b) Huh7_cont and Huh7_HBx cells were transfected with cont shRNA or TBK1 shRNA and then cell viability was determined using a CCK-8 assay. (c) Hep3B cells were co-transfected with various combinations of pRcCMV/HA-HBx, cont shRNA or TBK1 shRNA and then cell viability was determined using a CCK-8 assay. The expression of TBK1, HBx and β-actin in Hep3B cells, was examined by immunoblot analysis with anti-TBK1, HA and β-actin antibodies. (d) TBK1 and β-actin levels in HCC tissues. Immunoblot analysis was performed using anti-TBK1 or β-actin antibodies. T, tumor; N, non-tumor; G, Edmondson–Steiner's grade. (e) The extent of increase in TBK1 level correlated with histological grade. *P* value, Spearman correlation.*, Value was statistically significant. *N*, number of cases.

hepatocellular carcinoma tissue samples. These findings suggest that HBx-mediated regulation of TBK1 occurred in both our HBx-expressing cell system and in patient tissues (Fig. 4). Thus, TBK1 may play a role in regulating the HBx-mediated NF-κB pathway during the development of hepatocellular carcinoma.

Previous reports have identified various mechanisms by which HBx is able to mediate NF-κB activity. Previously, we found that HBx was able to up-regulate the expression of Cyclin D1 via the NF-κB-binding site in the Cyclin D1 promoter. In that report, we also proposed that HBx was able to up-regulate BCL-3, which forms a complex with p52 to activate the transcription of Cyclin D1 [16]. Our RT-PCR array analysis detected an increase in BCL-3 in the HBx-expressing cell line, which confirms the findings in our previous report. However, we were able to identify a much greater increase in TBK1 levels, which prompted our selection of TBK1 as the focus for this study. As a multifunctional protein that is capable of activating several key signaling pathways, it is not too surprising that HBx is able to activate NF-κB through different pathways. The fact that HBx has the ability to regulate several genes that may in turn activate NF-κB signaling highlights its importance in NF-κB activation.

However, there were some differences between our findings and previous reports, specifically in regard to TNFα [17] and interferons, which are key proteins in NF-κB signaling. One reason for these discrepancies may be the use of different cell systems. To observe up-regulation of TNFα, Pezzi et al. performed a transient transfection of HBx into HepG2 cells. In this report, we used a stable cell line derived from Huh7 cells. Furthermore, differences in gene regulation may have resulted from growth selection in our HBx cell line.

The role of IKKε in NF-κB activation has not been fully elucidated. In a previous study, it was reported that IKKε-/- cells show unchanged inducible IκBα phosphorylation [18]. Therefore, HBx/TBK1 activation of NF-κB might be independent of IKKε.

In this report we have shown that HBx-mediated up-regulation of TBK1 expression may contribute to HBx activation of NF-κB. We also found a positive correlation between the expression level of TBK1 and hepatocellular carcinoma. Therefore, we propose that HBx regulation of TBK1 may induce the development of hepatocellular carcinoma that is observed in the HBV-positive population. Our findings may suggest new therapeutic approaches for curing hepatocellular carcinoma by targeting TBK1.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.11.091.

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